RESEARCH ARTICLE

Production of Keratinolytic Proteases from Horn Meal by Newly isolated *Bacillus* sp.

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ABSTRACT: Keratinase are proteolytic enzymes that have gained much attention to convert keratinous wastes that cause huge environmental pollution issues. A new proteolytic *Bacillus* sp. was isolated from tannery effluent waste soil sample. The bacterium produces extracellular keratinase using horn meal as a sole carbon and nitrogen source. The effect of pH, temperature, and substrate concentration on extracellular keratinase production of this proteolytic strain was studied. Maximum enzyme productions were obtained 681U/mL for 72 hrs of cultivation. The optimal conditions for the proteolytic activity were determined to be pH 8.0 and temperature 40 °C; however the proteolytic activity showed a broad range of pH between 6-10 and temperature 30 °C – 50 °C. The zymogram showed hydrolysis activities of gelatin around the 28 kD. These results indicate that this *Bacillus* sp. shows a high potential for keratinase production.

KEYWORDS: Keratin, Horn meal, Protease, *Bacillus* sp., Zymogram.

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1. INTRODUCTION

Keratin is the most plentiful structural protein in skin, hair, wool and feathers. Keratins are proteins that form hard fibers and are components of the epidermal and skeletal tissues. Disulphide and hydrogen bonds make keratin a stable protein resistance to proteolytic hydrolysis [1, 2]. Keratinases (E.C. 3.4.99.11) belong to the group of serine hydraulics that are accomplished of mortifying keratin, a fibrous and insoluble structural protein extensively cross linked by disulfide, hydrogen and hydrophobic bonds [3].

Keratinases are a group of mostly extracellular serine proteases that are able to degrade keratins, a group of fibrous, insoluble and abundant structural proteins that constitute the major components of structures such as hair, wool, nails, hooves, horns and feather quills. Large amounts of keratin-containing wastes are discharged every year by the poultry, leather and meat processing industry. Current estimates indicate that the global annual discharge of feather from poultry processing industries alone reaches millions of tons [4, 5].

Microbial proteases represent one of the three largest groups of industrial enzymes and they have extensive applications in a range of industrial and household products including detergents, food, leather, silk and in pharmaceutical industries [6, 7]. Microbial keratinase are a class of proteolytic enzyme capable of degrading the insoluble structural protein found in feathers, hair, and wool known as keratin. This protein is resistant to degradation by common proteolytic enzymes such as trypsin, pepsin, papain due to the composition and molecular conformation of the amino acids found in keratin [8, 9].

Microorganisms represent attractive sources of proteases, as they can be cultured in large quantities by establishing methods in a relatively short time, producing an abundant and regular supply of the enzyme. Among microbial sources, representatives of the bacterial genus Bacillus are recognized as good protease producers and, on the commercial scale, proteases are generally produced by submerged fermentations [10, 11]. *Bacillus* strains are known to produce and secrete large quantities of extracellular enzymes and constitute a major group of industrial enzyme producers due to the robust nature of the organism as well as its enzymes. Several *Bacillus* isolates produce extracellular proteases which act on feathers, e.g., *Bacillus licheniformis* [12], *Bacillus subtilis* [13], *Bacillus cereus* [14], *B. pseudofirmus* [15], and others [16]. Likewise, many Bacillus strains secrete keratinolytic proteases that are useful in dehairing of animal hides, for instance *B. subtilis* [17, 18], *Bacillus velesensis* [19], *B. cereus* [20], *B. licheniformis* [21], and *Bacillus pumilus* [22]. Other potential groups of keratinolytic organisms include *Streptomyces* sp., dermatophytic fungi such as *Trychophyton* sp. and *Microsporuim* sp. and *archaea* belonging to *Crenarchaeota* [23].

Horn meal contains alpha keratin. Its micro fibrile are about 75 A° in diameters. It is also called as scleroprotein. Keratin are formed in the outermost living cells of the skin, they are structural protein of hair, wool, feathers, horn, nail and hoofs. The cystine seems to be fundamentally involved in the insolubility of keratin and resistance to enzymes. Keratin is insoluble in water, aqueous solution of neutral salts and organic solvents. The postulated basic polypeptide subunit of keratin has a molecular weight of approximately 10,400.The disulfide bonds of keratin stabilize the stereo chemical configurations that are responsible for the resistance of these substances to chemical and enzymatic attack. Upon destruction of the disulfide bonds, keratin becomes easily

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denatured and then loses its natural insolubility and resistance to proteases

In the present work, the production of extracellular keratinases by *Bacillus* sp. newly isolated from tannery effluent soil was investigated. Considering that the presence of keratinous substrates usually induces keratinase production, the main aim of the study was horn meal on the production of keratinolytic enzymes by *Bacillus* sp.

2. MATERIALS AND METHODS

2.1. Sample collection

A sample collected from tannery effluent soil, Trichirappalli, Tamil Nadu, India and also sample collected from horn meal preparing the area and soil sample collected from the slaughter house.

2.2. Substrates

Horn meal was collected from the tannery industries and washed twice with double distilled water to eliminate the irregular dust and soil from the material and dried at 60 °C overnight. It was kept at room temperature and used as raw material.

2.3. Isolation of proteolytic organisms

Microorganisms were isolated from different samples was suspended in 10mL of physiological saline solution (10^{-1}) and mixed vigorously by using a cyclomixer. From this solution, the aliquots were serially diluted up to 10-7 dilution. From each dilution 1mL of the aliquots was transferred to approximately marked skim milk agar plates.

2.4. Screening on skim milk agar plates

Skim milk agar (Himedia) was prepared and the above dilutions were streaked on milk agar plates for testing the caseinolytic activity of the organism. Bacteria were inoculated onto plates and incubated at 37 °C for 24 hrs. Strains that produced clearing zones in this medium were selected [24].

2.5. Morphological studies of isolated bacterial strains

Bacterial identification was conducted on morphological, physiological and biochemical tests. Results were compared with Bergey's Manual of Determinative Bacteriology, 8th edition [25]. The strain was also identified by a chromogenic method on the *Bacillus* differential agar from Himedia M1651 (India) optional for fast identification of *Bacillus* sp. from a mixed culture.

2.6. Media optimization for keratinase production

The sterile mineral salt liquid medium 100mL was inoculated with 5mL culture suspension of the isolated *Bacillus* sp. (0.1 O.D. at 520nm) and were incubated in shaker incubator at 37 °C for 48 hrs. Static condition was maintained as control. To check the effect of pH each flask of 100mL medium adjusted to different pH such as 6, 7, 8, 9 and 10 using 1 N NaOH and 1 N HCl. To study the effect of temperature each flask was incubated at different temperature (30, 35, 40, 45 and 50 °C) each temperature was separately taken in a conical flask and maintained pH 8.0, and sterilized at 121 °C for 15 min. Then 5mL of seed culture was inoculated into the mineral salt medium incubated for 72 hrs. The effect of concentration on keratin degradation was tested by adding different concentration of horn meal keratin such as 0.5, 1, 1.5, 2 and 2.5%, maintained pH 8.0, temperature 37 °C sterilized at 121 °C for 15 min. Then 5mL of seed culture was inoculated into the horn meal salt medium incubated at 40 °C.

2.7. Partial purification of extracellular keratinase enzyme

The crude extract fluids (100 mL) were precipitated by 70% saturation using ammonium ion salt. The addition of salt was through with constant stirring on a magnetic stirrer in an ice tub. The protein precipitate obtained was separated by centrifugation at 10,000 rpm for 10 min and also the pellet was dissolved with minimum volume of Tris-HCl buffer pH 8.0. The dissolved sample was dialyzed (Cellophane membrane, Himedia) against Tris-HCl buffer pH 8 for 8 hrs. The dialyzed enzyme was collected and assayed for its enzyme and protein activity.

2.8. Assay for proteolytic activity

Casein of concentration 1% (w/v) as a substrate dissolved in 20mM Borate buffer pH 9.0 containing 2mM $CaCl_2$. The proteolytic activity of the mixture containing 2mL casein, 1mL of the enzyme and the enzyme reaction was stopped with 2.5mL 0f 10% TCA, then incubated at 45 °C for 30 min and filtered using Whatman No.1 filter paper. To 1mL of filtrate, 5mL of 0.55M sodium carbonate solutions and 0.5mL of Folin-Ciocalteau's reagent (1:2) were added and keep at room temperature for 30 min. The absorbance was measured at 640nm. Simultaneously a blank was run using the same steps, except the addition of enzymes. The results were expressed in the amount of protease unit (U) per mL of enzyme extracted per unit time [26]. One protease unit is defined as the amount of enzyme required for producing 1µg of tyrosine/mL of enzyme in 30 min at 45 °C. Using the standard graph, the enzyme unit was calculated.

2.9. Protein determination

Protein concentration of the enzyme preparations were determined according to the method described by Bradford [27], using bovine serum albumin as the standard.

2.10. Zymogram of keratinase

Zymogram studies were performed using 0.2% (w/v) gelatin as substrate co-polymerized with 12% resolving gel and 4% stacking gel. Purified protein was mixed with sample buffer and loaded to polymerized gel without any heating. After electrophoresis, the gels were divided in two parts. A part was washed in 2.5% (v/v) Triton X-100 for 30 min to remove SDS and allow the renaturation of proteases. The gels were then incubated in phosphate buffer for 15 hrs at 37 °C and stained with Coomassie brilliant blue (CBB) R-250. Another part was transferred into 1% casein and incubated for 30 min at 37 °C in the presence of 50 mM tris buffer with pH of 8.0. Following incubation, the gel was stained with CBB G-250 to visualize the hydrolysis zones [28].

3. RESULT AND DISCUSSION

3.1. Selection and identification of micro-organisms

Samples collected from different areas were tested on

skimmed agar plates to detect the protease producing strains. By this, bacterial strains exhibiting clear zones of casein hydrolysis in the region of their colonies were chosen for keratin degradation activity in liquid medium. Morphological examinations and biochemical tests was done for the identification of keratin degrading organism.

Table 1 presents morphological and biochemical characteristics of the isolate. These results were compared with the organism given in the Bergey's manual. According to these results, the strain was identified as *Bacillus* sp.

Table 1: Morphological and Biochemical characteristics of *Bacillus* sp.

TEST	RESULT
Morphology	
Gram staining	Gram positive
Spore staining	Spore forming
Motility test	Motile
Biochemical test	
Catalase	+ve
Oxidase	+ ve
Coagulase	+ve
Indole	-ve
Methyl red	+ve
Voges prosakeur	+ve
Citrate utilization	+ve
Carbohydrate fermentation	
Mannitol	-
Lactose	-
Sucrose	+
Fructose	+
Dextrose	+
Triple sugar iron	Acid butt, Alkaline slant,
Starch hydrolysis	+
Urea hydrolysis	+

3.2. Effect of culture conditions on enzyme production

The effect of culture condition was found to play an important role on the keratin degrading activity by *Bacillus* sp. The optimal pH, temperature and the substrate concentration were calculated from the protease assay and protein content in the filtrate on different incubation period (Table 2, and Fig. 1 and 2). The percentage of hydrolysis was given in the fig 3.

3.2.1. Effect of pH

Protease activity, protein content and the percentage of keratin hydrolysis was found to be maximum in pH 8.0. It was found to be optimum pH for keratin degradation by *Bacillus* sp. But the activity was found to be less under acidic conditions at pH 2 and similarly the activity slowly decreased with increasing pH. The maximum Protease activity and protein content at pH 8 was found as 640.31 U/mL, 3.36 mg/mL respectively on day 3. The percentage of keratin hydrolysis at this pH was 92% and it was comparatively less at other pH on day 5.

3.2.2. Effect of temperature

Protease activity, protein content and the percentage of keratin hydrolysis was found to be maximum at 40 °C. It was found to be optimum at 40 °C for keratin degradation by *Bacillus* sp. But the activity was found to be less in other temperatures. The maximum Protease activity and protein content at 40 °C was found as 681.79 U/mL, 3.30 mg/mL respectively on day 3. The percentage of keratin hydrolysis at 40 °C was 96% and comparatively less at other temperatures on day 5.

3.2.3. Effect of substrate concentration

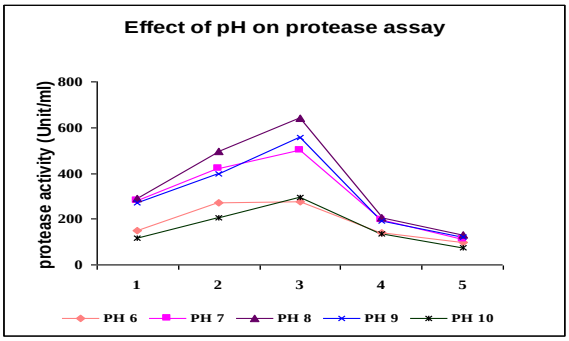
Protease activity, protein content and percentage hydrolysis was found to be maximum in 1% substrate (keratin) concentration. It was found to be optimum concentration for keratin degradation by *Bacillus* sp. Keratin concentration affects bacterial growth and keratin degradation. Bacterial growth increased as the keratin concentration increased, while the percentage of keratin degradation was inversely proportional to the keratin concentration. High keratin concentration may cause substrate inhibition or repression of keratin degradation, resulting in a low percentage of keratin degradation. The maximum Protease activity and protein content at 1% concentration was found as 556 U/mL, 3.81 mg/mL respectively on day 3. The percentage of keratin hydrolysis at this concentration was 94% and comparatively less on other concentrations (Fig. 3).

Table 2: Protease assay (U/mL) at different pH, Temperature and concentration

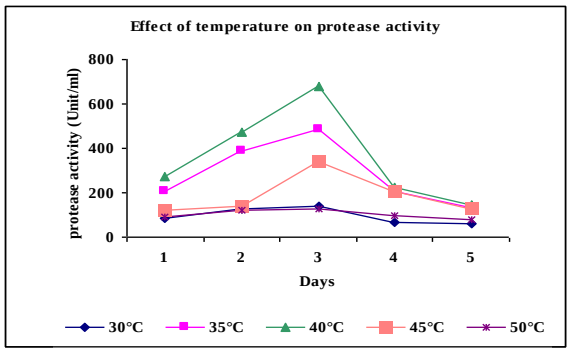
Factors	Time (Hrs)				
	24	48	72	96	120
pH					
6	151.72	271.83	277.93	138.48	99.90
7	282.71	420.97	501.26	197.05	114.37
8	290.14	496.28	640.31	208.07	132.28
9	271.00	399.28	556.88	191.54	121.95
10	118.00	207.38	292.85	136.42	75.79
Temperature (°C)					
30	82.68	129.53	136.42	69.58	63.38
35	205.32	387.24	487.19	208.07	132.28
40	271.47	472.54	681.79	225.87	144.69
45	118.50	138.48	339.17	204.45	125.39
50	90.94	121.95	129.15	97.83	75.79
Concentration (%)					
0.5	122.60	319.54	366.93	206.70	151.58
1.0	237.06	387.24	556.00	291.11	177.76
1.5	156.55	270.80	390.62	285.69	137.80
2.0	137.80	272.15	382.66	291.78	150.20
2.5	136.42	252.52	367.61	271.47	148.13

Fig. 1

(a) Effect of pH on protease activity



(b) Effect of temperature on protease activity



(c) Effect of Substrate concentration on protease activity

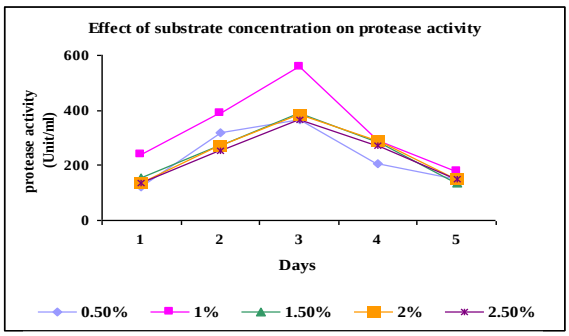
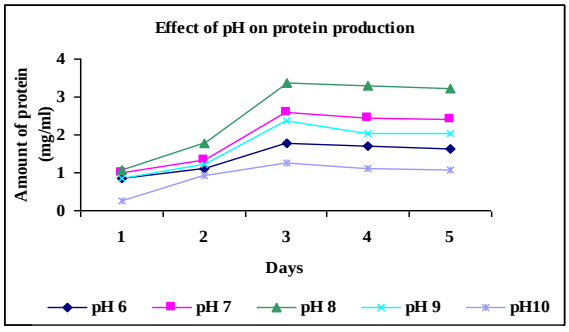
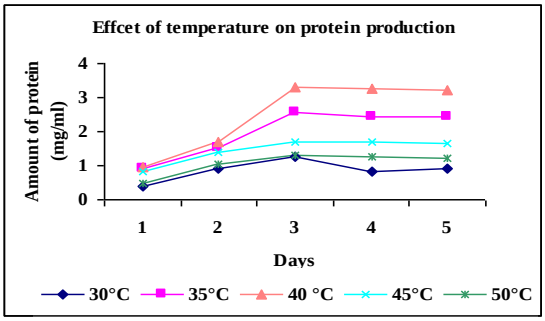


Fig. 2

(a) Effect of pH on protein production

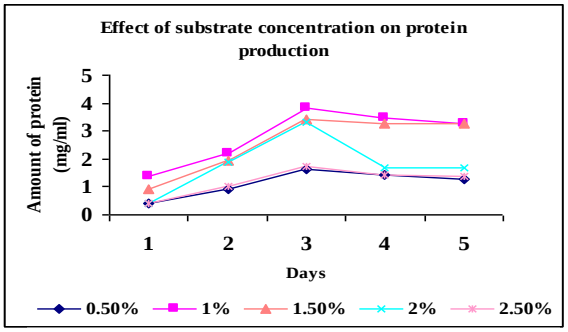


(b) Effect of temperature on protein production

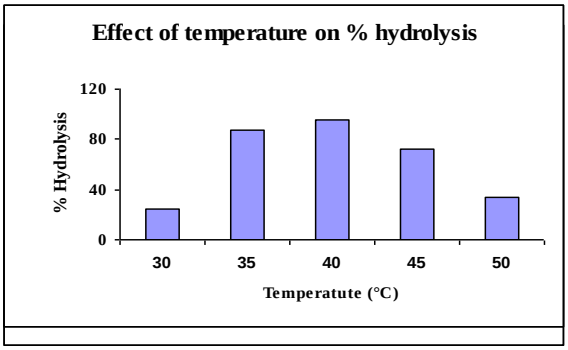


(c)Effect of Substrate concentration of Protein production

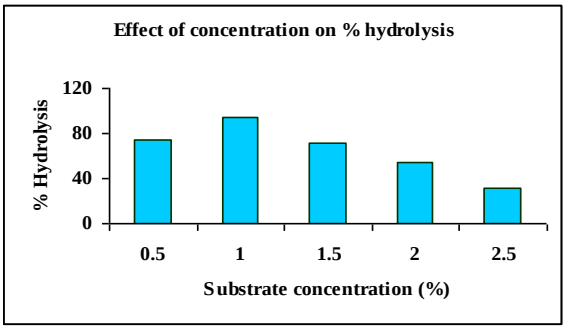
Fig. 3



(a) Effect of pH on % Hydrolysis



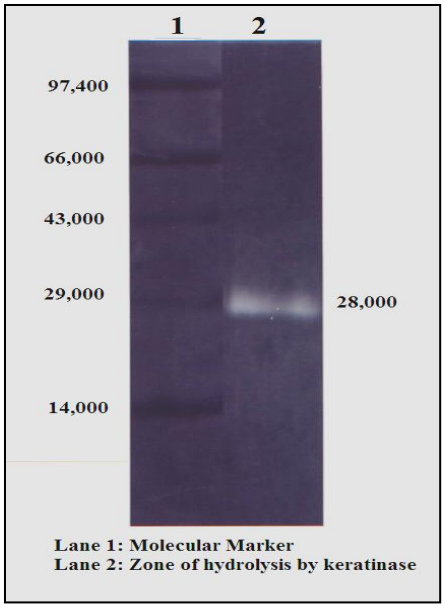
(b) Effect of temperature on % Hydrolysis



(b) Effect of Substrate concentration on % hydrolysis

3.3. Gelatin zymogram

The eletrophoretic separation of the purified enzymes at 4 °C on gelatin zymogram revealed a band of proteolytic activity as seen as clear area (Fig.4). Zone of hydrolysis observed in the gelatin zymogram gel, indicated keratinase activity of the purified enzyme (28kD).



Lane 1: Molecular Marker
Lane 2: Zone of hydrolysis by keratinase

The isolated bacterium showed high proteolytic activity when cultured in substrate medium. Optimum proteolytic activity was observed at 40 °C (681.79U/mL). It is also stabled at pH 8.0 (640 U/mL) [29].

The most favorable temperature of protein production is ranging from 30 to 80 °C. Nam *et al.,* [30] showed exceptionally high temperature optima of 90 and 100 °C, respectively, with a half-life of 30 and 90 min respectively. The pH and temperature stability indicates that keratinases are generally active and stable over a wide range of pH from 5 to 13 [31]. *Bacillus* strains are ubiquitous microorganisms, which can grow on natural media without any special requirements. These properties can be exploited in the degrading feathers, which are produced in huge amounts throughout the world.

Microbial proteases possessing keratinase activity may be an alternative eco-friendly strategy to replace the use of ecohazardous chemicals for the dehairing purpose. However, most of the proteases may be unsuitable for this process because of the associated collagen-degrading activity which reduces the leather quality [32]. Absence of collagenase activity in Brevicarnase suggested its commercial exploration in leather industry for dehairing purpose.

4. CONCLUSION

Horn meal (HM) was the low-cosat waste residues, best growth substrate for production of extracellular keratinolytic proteases by newly isolated *Bacillus* sp. The zymogram showed hydrolysis activities of gelatin around the 28 kD. Such microbial enzymatic technology represents a valuable approach with the advantage of recycling of agro-industrial residues and associated aggregation of value to these inexpensive raw materials.

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